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STABILIZED LACCASES AS HETEROGENEOUS BIOELECTROCATALYSTS
POSTPRINT

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14. ABSTRACT

Typically, heterogeneous enzyme catalysis is aimed at sustainability, reusability or enhanced functionality of the biocatalyst and is achieved by immobilizing that enzyme to a support matrix or at a defined interface. Controlled enzyme immobilization is particularly important in bioelectrocatalysis because catalytic activity must be effectively coupled with a transducer in order to harness activity. This review discusses what must be addressed when coupling biocatalysts with a transducer and the toolbox of methods available to achieve this outcome. As illustration, the focus here is the immobilization and stabilization of laccases at the electronics interface. Historically, laccases have been used for the decolorization of dyes and for bio-organic synthesis, but recently have been applied in the fields of sensing and energy harvesting.[1-3] There is ever increasing focus directed towards new energy technologies, for example, in which laccases find application as cathodic catalysts in enzymatic fuel cells. Herein, we discuss the steady advancement over the past 10 -15 years in using heterogeneous laccase biocatalysts and demonstrate why laccases continue to be biotechnologically relevant enzymes. The methodologies for immobilization of laccases are described including the use of nanoscale supports and a range of encapsulation and cross-linking chemistries. We consider the application of immobilized laccases to the food industry, pharmaceutical synthesis and environmental applications, specifically where stabilization through heterogenization of the enzyme is critical to the application. We also include a consideration of electrochemical biosensors and the specific incorporation of laccases at transducer surfaces.

15. SUBJECT TERMS

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Stabilized Laccases as Heterogeneous Bioelectrocatalysts

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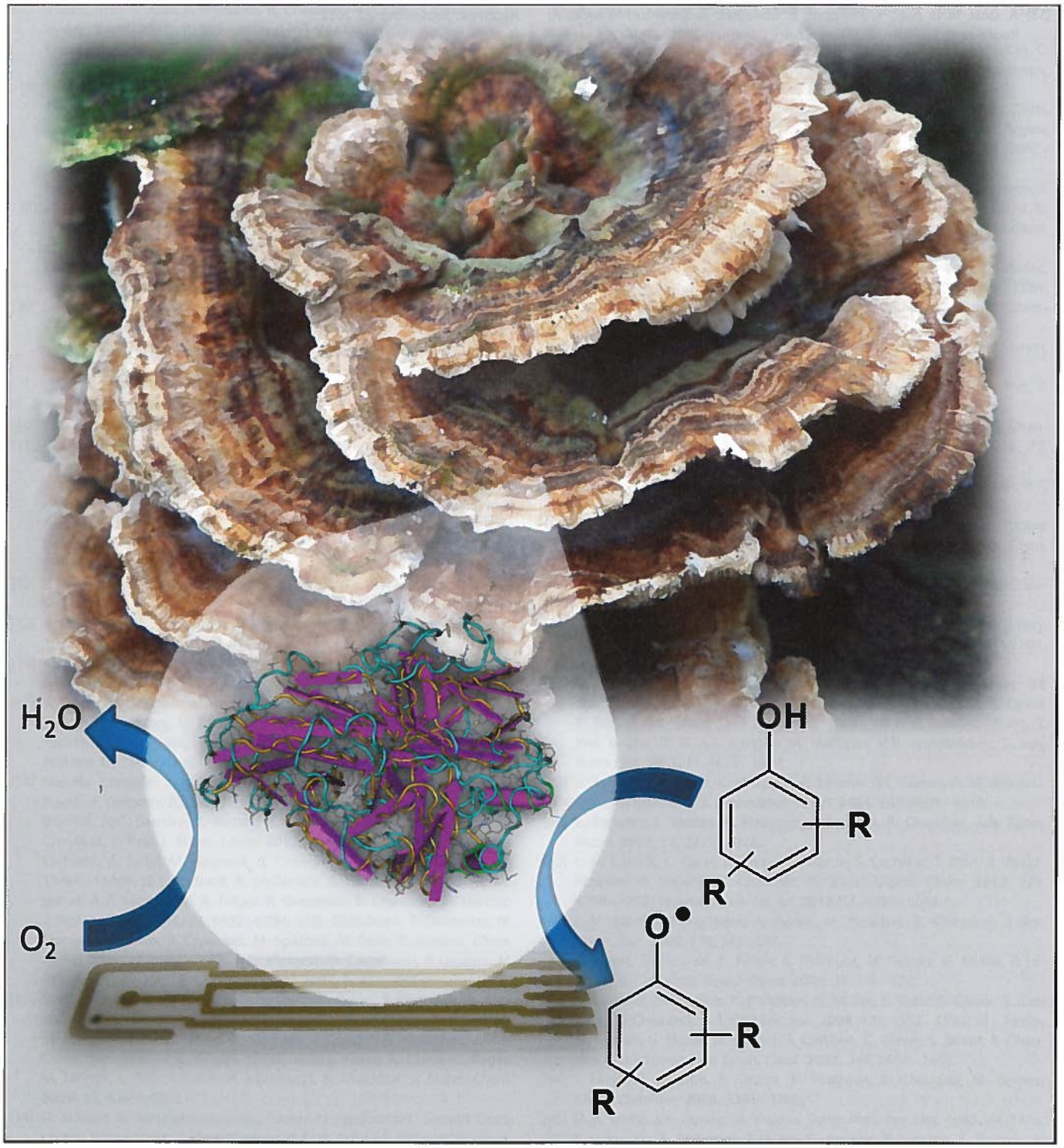

Typically, the use of heterogeneous enzyme catalysis is aimed at sustainability, reusability, or enhanced functionality of the biocatalyst and is achieved by immobilizing enzymes onto a support matrix or at a defined interface. Controlled enzyme immobilization is particularly important in bioelectrocatalysis because the catalyst must be effectively connected to a transducer to exploit its activity. This Review discusses what must be addressed for coupling biocatalysts to an electrode and the toolbox of methods that are available for achieving this outcome. As an illustration, we focus on the immobilization and stabilization of laccases at electronic interfaces. Historically, laccases have been used for the decolorization of dyes and for the synthesis of bio-organic compounds; however, more recently, they have been applied to the fields of sensing and energy harvesting.[1–3] There is an ever-increasing focus on the development of new energy technologies, in which laccases find application (e.g., as cathodic catalysts in enzymatic fuel cells). Herein, we discuss the heterogeneous laccase biocatalysts that have been reported over the past 10–15 years and discuss why laccases continue to be biotechnologically relevant enzymes. Various methods for the immobilization of laccases are described, including the use of nanoscale supports and a range of encapsulation and cross-linking chemistries. We consider the application of immobilized laccases to the food industry, in the synthesis of pharmaceuticals, and in environmental applications, specifically in cases in which stabilization through heterogenization of the enzyme is critical to the application. We also include a consideration of electrochemical biosensors and the specific incorporation of laccases on the surfaces of transducers.

1. Introduction

Enzyme diversity has provided biotechnology with a wealth of catalysts from which man has benefited enormously. Drug discovery, the preservation and preparation of food, the decontamination of toxic compounds, and chemical detection are only a few examples of the multiple fields in which enzymes are used as biotechnological tools.[4–6] Among the biotechnologically relevant enzymes, laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) stand out owing to their wide catalytic versatility, physiological variability, and good process stability.[7,8] As such, examples of laccase biocatalysis have been demonstrated in applications that range from bio-bleaching to biotransformation and biosensor technology.

Laccases are multicopper oxidases that are found throughout nature, with examples that range from bacterial species to fungi and plants.[9,10] However, certain properties of laccases, such as their protein structure, redox potential, and the organization of the catalytic copper atoms, vary significantly across the *phyla*. Many forms of laccase have been identified, including examples of extremophilic enzymes,[11,12] but the extracellular laccases from wood-rotting fungi, such as *Trametes* spp., *Cerrena maxima*, and *Rhus vernicifera*, are by far the most commonly studied in bioelectrocatalysis.[9,13,14]

The redox potential of the multicopper active site is intricately linked to the substrate specificity of the protein and its ability to oxidize phenolic substrates, which is thermodynamically driven by the concomitant reduction of molecular oxygen.[5] In nature, laccases are able to oxidize various complex electron donors in the presence of chemical mediators that act as electron shuttles, diffusing between the enzyme and the donor to facilitate the oxidation of large polymeric molecules, such as lignin.[15,16] Typically, owing to their inherent redox potential (0.5–0.8 V versus Ag/AgCl), laccases are unable to oxidize non-phenolic lignin units that have a high redox potential (> 1.5 V). However, in the presence of mediators, the oxidation of non-phenolic substrates becomes feasible and, as such, laccase-mediated systems have found utility in environmental and bioremediation applications, specifically in pulp delignification and in the oxidation of organic pollutants.[17]

The architecture of the laccase active site consists of a high-potential type-1 (T1) copper atom, which is located near the substrate-binding pocket, and a cluster of three copper atoms (type-2 (T2) and type-3 (T3)), which are buried further inside the protein structure (Figure 1). The T1 copper atom functions as the primary electron acceptor and shuttles electrons to the T2/T3 copper cluster and dioxygen is fully reduced into water without the release of a H_2O_2 intermediate.[15] Laccases all contain at least one T1 atom and a T2/T3 trinuclear copper cluster, which is typically located in the active site in a rhomboid fashion (Figure 1). The T1 copper atom exhibits a strong absorption band at around 600 nm, which gives rise to a characteristic blue color; as a result, many of these multicopper oxidases are also termed "blue" copper oxidases.[8] The unique spatial distribution of the copper atoms in laccases, as well as their constrained coordination in the folded protein, are, in part, responsible for the good electron-transfer properties of these enzymes.[8] The electron-transfer efficiency of laccases in response to oxidation of the substrate facilitates their application in bioelectrochemical sensing.

To date, laccases have been broadly used to detect phenolic compounds,[18,19] catecholamines,[4] biopolymers,[20] small aromatic compounds (such as cysteine),[6] and to measure glucose concentration.[21] As the research into laccase biosensors has developed, it has become clear that, as in the case of other ox-

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idoreductase-based biosensors, the integration of biomolecules into transducers is critical for obtaining good biosensor performance. In this respect, recent methods for the integration of laccases into various materials have resulted in a rapid growth in the development of biosensors for analytical processes (Table 1). In-fact, reports of laccase-based biosensors have grown significantly over the last five years, as judged by the number of publications in this field (Figure 2).

The heterogenization of a sensing molecule through its attachment onto an electrode surface is a key aspect in the development of enzyme-based electrodes. The immobilization of biomolecules not only affects the stability and selectivity of an enzyme towards different substrates but also the charge transfer at the electrode surface.[22] This limitation is usually addressed by using a range of immobilization strategies that involve different interactions (covalent, ionic, metal chelation, etc.) between the protein and the transducing element and pre- and post-immobilization techniques, such as microencapsulation and cross-linking (Figure 3).[23] The use of nanoscale supports has also enabled a remarkable improvement in biosensor sensitivity through an increase in biocatalyst loading. Moreover, emerging technologies have enabled the use of more effective transduction methods that allow the detection of very small amounts of analyte and also effectively detect recognition and binding events that occur on the transducer surface.

This Review will summarize the various heterogenization strategies and transduction methods that, taken together, provide a technological base for exploiting laccases as sensor molecules in detection devices. Special attention will be paid to the details of surface functionalization and pre- and post-immobilization strategies that affect the stability of the biosensors, as well as the transducer materials that improve sensor design.

Lorena Betancor studied at the Universidad Autónoma de Madrid and at the Institute of Catalysis in Madrid with a focus on enzyme stabilization, protein modification, and the design of chromatographic supports for biocatalysis. Her postdoctoral research with Prof. J. Spain at Georgia Tech (USA) and with Prof. P. Leadlay at the University of Cambridge (UK) focused on the enzymology and molecular biology of nitroreductases, mutases, and polyketide synthases. She is currently an Associate Professor at the Universidad ORT Uruguay. Her research expertise involves the expression and purification of recombinant enzymes, the improvement of protein properties by immobilization, the enhancement of biodetection systems, and the in vitro use of multi-enzymatic systems.

Glenn Johnson earned his doctorate from the University of Michigan and has been part of the US Air Force Research Laboratory since 1995. He now leads the Microbiology and Applied Biochemistry Laboratory at Tyndall Air Force Base, Florida. His research interests include fundamental studies of biocatalysts, the development of biomaterials, and the use of those principles in diverse biotechnology applications. Specific developmental areas include enzymatic and microbial fuel cells, biosensors for environmental monitoring, and antimicrobial materials and coatings.

Heather Luckarift studied Process Biotechnology at the University of Teesside (UK), followed by obtaining her doctorate and undertaking postdoctoral studies in Biological Sciences at the University of Warwick (UK). Following a move to the US Air Force Research laboratory as a postdoctoral fellow in 2002, she is now a Senior Research Scientist in Microbiology and Applied Biochemistry. She has developed a series of niche technology areas in biocatalysis and protein stabilization for the development of antimicrobial biomaterials and biosensors. Her current research interests include biomimetic and biofunctionalized materials and the development of microbial and enzymatic fuel cells for biological energy conversion.

2. Applications of laccases

Laccases have found various applications, from environmental processes, such as delignification,[24] decolorzation, and the treatment of waste streams,[25] to biocatalysis, such as the production of antibiotics and food processing.[26,27] Laccases are widely used in bioremediation studies, owing to their ability to degrade phenolic compounds that constitute a significant health hazard in water sources.[28] This property is particularly useful for treating tannin-rich wastewater, such as waste from beer processing, which is high in polyphenols and is a distinctive and unattractive dark-brown color. The decolorization of such waste streams is an important treatment step, as well as decreasing the overall chemical demand for oxygen. As a result, commercial preparations of immobilized laccases are available for large-scale bioremediation processes. Laccases have found significant application in the textile industry, as well as commercial success in bio-bleaching and waste-stream remediation. Another widely used large-scale laccase-catalyzed bioremediation process is the polymerization of phenolic compounds in waste streams; for further information on laccases for biodegradation, the reader is directed to other recent reviews.[3,29]

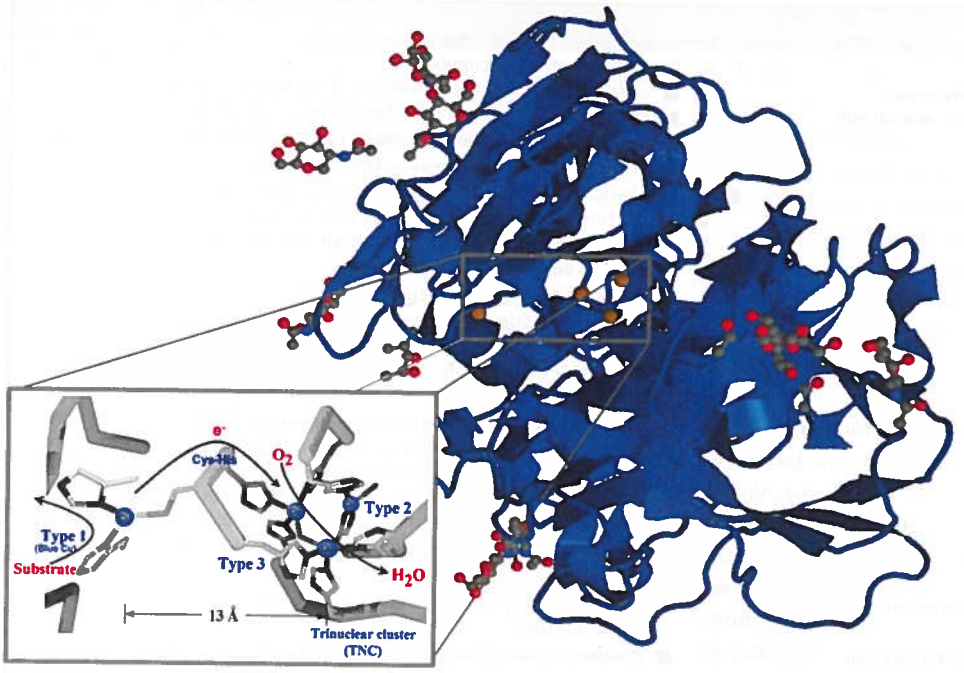

Figure 1. Structure of *Trametes versicolor* laccase from the RCSB protein data bank (1GYB); copper atoms that form the active site are shown in orange. Inset: Distribution of copper atoms T1 and T2/T3 and representation of the reaction catalyzed by laccases; arrows mark the flow of the substrates, electrons (e^-), and O_2. Reproduced with permission, RSC publishing, 2008.[104]

2.1. Food processing and quality

In food processing, laccases are often used to catalyze reactions that enhance the appearance of food and beverages.[27] For example, specific interactions between polyphenols and residual proteins from fruit harvesting lead to precipitates that cloud fruit juices; laccases help to polymerize the phenols, thereby simplifying their removal before they foul stored juices. The same issue confounds beer makers and it can be addressed by adding laccase directly to the wort (unfermented malt).[30] Adding laccases at the end of the brewing process also helps to remove oxygen, which improves the storage stability of the beer. Similar processing is used to improve the quality of edible vegetable oils by removing residual

Table 1. Methods for laccase immobilization with applications in biosensors. Immobilization method: Adsorption (A), encapsulation (E), covalent (C).

Laccase source	Biosensor	A	E	C	Immobilization description[a]	Reference
Agaricus bisporus	dopamine			■	covalent immobilization (EDC/NHS) onto a mercaptopropionic acid SAM on Au	[105]
Aspergillus oryzae	catecholamines and methyldopamines	■			physical adsorption: cellulose-acetate/ionic-liquid/carbon-paste	[46]
	epinephrine	■			physical adsorption: ionic-liquid/carbon-paste composite	[106]
	rutin content of pharmaceuticals	■			physical adsorption: graphite-powder/hydrophobic-ionic-liquid	[107]
	methomyl (pesticide)	■			physical adsorption: Pt Nps in ionic liquids	[108]
	catecholamines	■			physical adsorption: Pt Nps in ionic liquids	[4]
	L-cysteine	■			physical adsorption: graphite powder in a carbon paste	[6]
	methomyl in vegetable extracts	■			immobilization onto sol–gel-derived ceramic carbon	[53]
	rutin		■		entrapment in chitosan that was cross-linked with Na tripolyphosphate	[109]
	luteolin in tea			■	Ag/Au nanoparticles in ionic-liquid/chitosan that was cross-linked with $(NCCl)_3$	[107]
	rosmarinic acid in plant extracts	■			laccase in graphite powder, mineral oil, and $[BMi]PF_6$	[110]
Cerrena unicolor	phenolic compounds and catecholamines	■			physical adsorption: graphite electrode	[111]
Coriolos versicolor	catechol		■		entrapment in a chitosan/CNT composite	[67]
	polyphenols in red wine	■			physical adsorption: polyethersulfone membranes	[38]
Ganoderma lucidum	polyphenols in beverages	■			physical adsorption: nitrocellulose-membranes/Pt-electrode	[112]
Ganoderma sp. Rckk02	polyphenols in beverages and pharmaceutical formulations			■	covalent immobilization: Cu-Nps/chitosan/MWCNT/PANI-Au	[72]
				■	covalent immobilization: Ag-Nps/MWCNT/PANI-Au	[113]
	phenolic compounds in tea extracts			■	covalent immobilization (EDC/NHS) of MnO_2 Nps on MWCNT/PANI/Au	[113]
	polyphenols in beverages		■		entrapment in cross-linked epoxy resins	[72]
Myceliophthora thermophila	pyrocatechol detection			■	glutaraldehyde cross-linking to graphite	[114]
Pleurotus ostreatus	catecholamines in pharmaceuticals	■			physical adsorption: graphite paste	[115]
Polyporus pinsitus	pyrocatechol			■	glutaraldehyde cross-linking onto graphite	[114]
Pycnoporus sanguineus	catechol, hydroquinone, resorcine	■			deposition onto a carbon-paste composite	[116]

Table 1. (Continued)

Laccase source	Biosensor	A	E	C	Immobilization description[a]	Reference
Rhus vernificera	catechin (anticancer flavonoid)			■	covalent binding (amide chemistry) to Au Nps in dendrimers	[82]
Rigidoporus lignosis	phenolic compounds in olive oil mill wastewater			■	covalent immobilization (EDC/NHS) onto a hydrophilic matrix	[85]
				■	covalent immobilization (EDC/NHS) onto a SAM on Au	[84]
Trametes hirsuta	catechol	■			physical adsorption: carbon ceramic electrodes	[43]
	polyphenols in wine	■			physical adsorption: polyazetidine pre-polymer on a CNT SPE	[36]
Trametes versicolor	chlorophenols		■		electrospun nanofibers: polyvinyl alcohol/surfactant, Au Nps	[117]
	polyphenols in wine		■		polyazetidine pre-polymer on MWCNTs and SWCNTs	[36]
	catechol	■			physical adsorption: carbon fibers	[118]
	caffeic acid in wines	■			physical adsorption: polyethersulfone	[34]
	catechol			■	1) glutaraldehyde cross-linking, 2) covalent binding with carbodiimide, 3) covalent binding onto carbon fibers with carbodiimide/glutaraldehyde	[118]
	phenolic compounds in herbal infusions			■	covalent binding to polyethersulfone membranes	[119]
	polyphenols in beer			■	cross-linking with glutaraldehyde: Nafion/Sonogel-C	[30]
	polyphenols in beer			■	cross-linking with glutaraldehyde: Nafion/Sonogel-C	[120]
	catechol			■	cross-linking through chitosan onto Cu-ordered mesoporous carbon	[121]
	phenolic compounds			■	covalent immobilization onto a 3-thienylmethyl/glycidyl-methacrylate co-polymer	[109]
	phenolic compounds and catecholamines			■	glutaraldehyde cross-linked enzyme aggregates with β-cyclodextrin	[122]
	phenolic compounds and guaiacol			■	covalent immobilization (EDC/NHS) onto acid-functionalized PTFE	[123]
			■		entrapment in gelatin through glutaraldehyde cross-linking	[123]
	polyphenols in wine			■	glutaraldehyde cross-linking onto glassy carbon electrodes	[37]
	phenolic compounds			■	cross-linked enzyme crystals	[122]
	azide and fluoride			■	glutaraldehye cross-linking to a colloidal clay suspension	[54]
	catecholamines in urine		■		alginate/enzyme suspension on an optical fiber	[103]
	catechol		■		immobilization in mesoporous silica on glassy carbon	[124]
	dopamine, norepinephrine		■		alginate/enzyme suspension on an optical fiber	[40]
	phenolic metabolites		■		entrapment during nanocomposite electrodeposition: chitosan/MWCNTs	[125]
	phenolic compounds in tea		■		entrapment in polyvinyl-alcohol/AWP on a carbon SPE	[126]
	xenobiotic compounds		■		entrapment in gelatin through glutaraldehyde cross-linking	[127]
	syringic, caffeic, and ferulic acids		■		entrapment in gelatin	[128]
	catechol	■			physical adsorption	[19]

Sources: Pubmed and Scopus searches of the terms "laccase AND biosensor" in abstracts only and limited to methods for single-enzyme heterogenization. Reports for biological fuel cells or biological oxygen reduction are excluded. [a] Abbreviations; PANI: polyanliline, MWCNT: multi-walled CNTs, SAM: self-assembled monolayer, PTFE: polytetrafluoroethylene, [BMI]NTf_2: 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, AWP: azide-unit-pendant water-soluble photopolymer, [BMI]PF_6: 1-butyl-3-methylimidazolium hexafluorophosphate, PEG: polyoxyethylene bis(glycidyl ether), SPE: screen-printed electrode, EDC/NHS: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride/*N*-hydroxysuccinimide, Nps: nanoparticles.

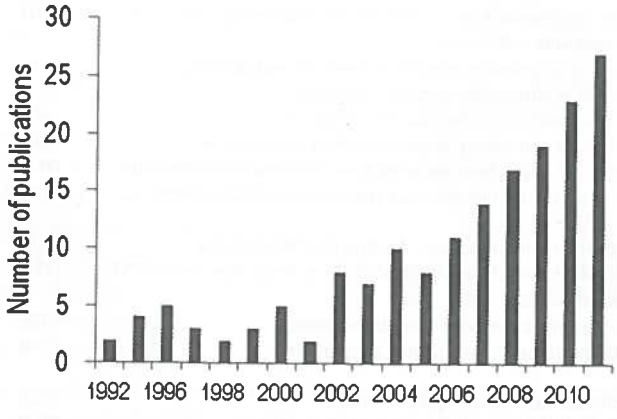

Figure 2. Number of publications that are related to laccase biosensors. Source: Scopus; retrieved April 2012, with "laccase" AND "biosensors" as the search criteria.

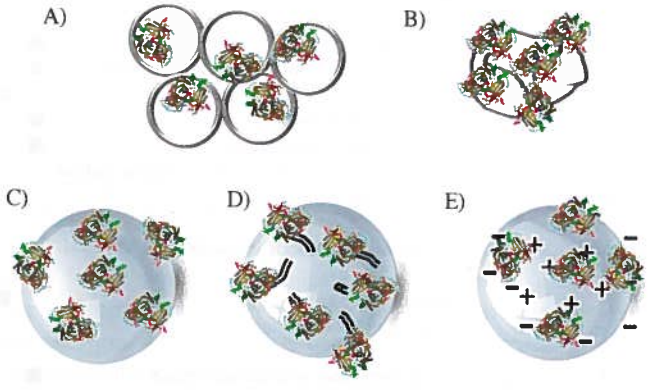

Figure 3. Typical methods for enzyme heterogenization, including A) encapsulation, B) covalent cross-linked enzyme aggregates, C) physical adsorption, D) covalent attachment onto a support, and E) electrostatic interactions.

oxygen that can lead to spoilage.[31]

The stabilization of wines is an area in which laccases have found specific utility. Alcohols and organic acids give wine its characteristic aroma, but its color and taste are largely determined by phenolic compounds, including cinnamic-acid derivatives, catechins, and anthocyanidins. However, the oxidation of phenolic compounds in musts (unfermented grape juice) and their subsequent interactions with residual metal complexes can cause turbidity and changes in flavor and ultimately cause wine to spoil. Recent reports have demonstrated that the moderate consumption of wine, particularly red wine, provides some health benefits that are attributed to the antioxidant properties of their polyphenol congeners.[32,33] Therefore, it is commercially advantageous that wine is stabilized to retain its characteristic "nose" during storage, but such treatments require careful consideration to ensure that the desirable polyphenol antioxidants are not removed. The primary mechanism to avoid spoilage is to remove polyphenols in the must by adsorption, filtration, or chemical modification. However, the oxidation of polyphenols by enzyme catalysis is an alternative (and much preferred) method of treatment for stabilization, owing to the selectivity of biological catalysts. The low optimum pH values of some laccase proteins are well-suited to wine processing and these proteins provide stabilization that is comparable to traditional processing methods. However, laccase is currently prohibited as a food additive; thus, its application necessitates the use of an immobilized form that allows its removal, thereby complicating widespread commercial use in food processing.[1,34,35]

However, the specific interactions between polyphenols and laccases can be used to monitor the quality of wine and beverages with respect to polyphenol content. Selective oxidation can be detected by incorporating laccases with electrochemical transducers to determine the polyphenol content in wine, tea, and vegetable extracts. The operating principle of a laccase-based biosensor of this type is the generation of current from the oxidation of phenolic compounds into quinones and radical species that are subsequently reduced at the electrode surface (Figure 4). This electronic method of detection has driven laccase immobilization on a range of solid electrode surfaces, including carbon fibers, carbon-black pastes, carbon nanotubes (CNTs), redox hydrogels, biomimetic membranes, and noble-metal electrodes.[36]

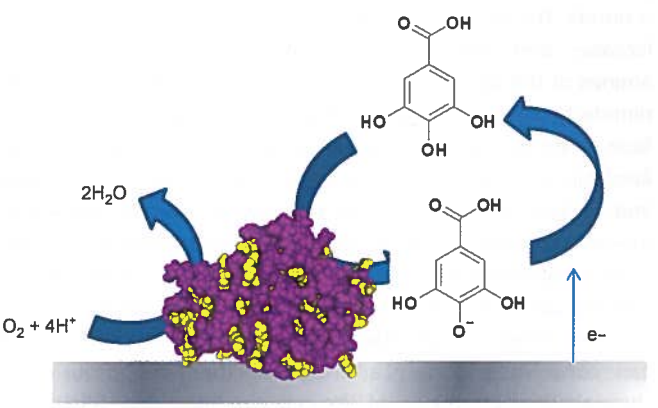

Figure 4. Electron-transfer mechanism for the oxidation of gallic acid on a laccase biosensor.

Initially, biosensors for monitoring the quality of wine relied upon the immobilization of laccases by using cross-linkers, such as glutaraldehyde, to fix the protein onto glassy carbon.[37] Similarly, laccases can be immobilized onto CNTs by functionalizing the CNT surface with a polyazetidine pre-polymer.[36] Such biosensors were used for the amperometric analysis of the concentrations of gallic and caffeic acid in a range of wines and provided comparable results to the standard (Folin–Ciocalteau) method for the determination of total polyphenol concentration.

Since these initial studies, the scope of polyphenolic analytes in red wine has been extended to caffeic acid, gallic acid, catechin, rutin, *trans*-resveratrol, quercetin, and malvidin by immobilizing laccase from *Coriolus versicolor* onto a platinum electrode through polyethersulfone membranes and using laccase activity as the sensing element of the process.[38] Discrimination of catechin from caffeic acid was achieved by the selective polarization of the electrode, but quantification and differentiation of the mixtures was difficult. This limitation is a common drawback of many biosensors, which cannot account for the influence of interfering organic compounds in actual samples. With genuine samples of wine, pre-isolation of the polyphenolic fractions by solid-phase extraction is often required for reliable detection and identification of the analytes.

2.2. Pharmaceutical analysis

The catechins found in wine are naturally occurring phenolic antioxidants that are derived from fruits and plants as secondary metabolites; they also exhibit a remarkable range of beneficial medicinal characteristics, including anticancer properties.[3,39] An interesting extension to the application of laccases in biosensors is the medically relevant detection of catechins and flavonoids, as well as catecholamines, such as dopamine and the human "fight or flight" hormones, which include epinephrine (adrenaline) and norepinephrine (noradrenaline).[40,41]

Various biosensors for the quantification of epinephrine, norepinephrine, and dopamine have been developed that use a range of laccase-immobilization techniques, including physical adsorption, covalent immobilization, and encapsulation. Comprehensive studies have been reported on the immobilization of laccases from *T. versicolor*, *T. hirsuta*, and *Cerrena unicolor* through physical adsorption onto solid graphite electrodes and their subsequent use in flow-injection analysis for the amperometric monitoring of phenolic compounds, including dopamine, L-DOPA, and epinephrine.[19,42,43] Typically, immobilization is directed at purified enzymes, but the retention of laccases in intact whole cells has also been demonstrated; for example, *Phanerochaete chrysosporium* mycelia were immobilized in gelatin by glutaraldehyde cross-linking onto a platinum electrode and demonstrated native laccase activity that was used as an amperometric biosensor for epinephrine.[44]

An alternative method for protein immobilization is the use of ionic liquids as the reaction medium for biocatalysis.[45,46] For

example, laccase from *Aspergillus ozyzae* was suspended with platinum nanoparticles in an ionic liquid that contained graphite powder to provide elements that could detect epinephrine at concentrations as low as about 300 nM.[4] One of the limitations of many epinephrine biosensors is interference from ascorbic acid. Ascorbic acid is oxidized at a potential that is close to that of epinephrine and is present in high concentrations in typical samples. To accurately measure epinephrine in the presence of ascorbic acid, Ou et al. fabricated a composite of CNTs in Nafion to limit the contact of laccase with ascorbic acid. Permeation of the positively charged conjugate base of epinephrine is enhanced by Nafion, whereas the permeation of neutral ascorbic acid is blocked; as a result, interference from ascorbic acid is effectively eliminated.[47]

Dopamine is a neurotransmitter that is intricately linked to many physiological and pathological phenomena. The electrochemical measurement of dopamine levels aims to provide real-time monitoring of cerebral dopamine, which is released during physiological and pathological processes.[48] Although recently developed nanomaterials, such as CNTs, may enable the effective electrochemical detection of physiologically important species, the structural similarity between dopamine and other catecholamines makes its selective detection challenging. However, Lin et al. reported the immobilization of laccase onto magnetite nanoparticles within a fused-silica capillary that was used to measure dopamine release in a cerebral microdialysate.[49] The biosensor takes advantage of a non-oxidative mechanism of laccase in which dopamine undergoes a series of sequential reactions to produce a quinone byproduct that can be electrochemically reduced. The byproduct is easily measurable and is directly proportional to the dopamine concentration. The same laccase-immobilized microreactor also eliminated interference from ascorbic acid and 3,4-dihydroxyphenylacetate, which are typically found in serum samples. The laccase oxidizes ascorbic acid and 3,4-dihydroxyphenylacetic acid into byproducts that are not electrochemically active and, thus, do not confound the target measurements.

Laccase-based biosensors can also be fabricated as bi-enzymatic systems, in which the laccases are co-immobilized with a second enzyme, typically tyrosinase or peroxidase, to amplify the sensor response.[50] For example, a distinction between morphine and codeine can be achieved by using a laccase-based bi-enzymatic biosensor. Morphine is oxidized by laccase with the consumption of oxygen and is regenerated by a second enzyme, pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase. In contrast, laccases cannot oxidize codeine, so the resulting biosensor is functionally selective for morphine.[51]

Rather than substrate turnover, biosensors can also be developed that rely on inhibition of the native catalytic activity.[52,53] For example, the inhibition of laccases by respiratory poisons, such as cyanide or azide, can be used as indicators for the presence of such toxins. As an example, laccase from *T. versicolor* was immobilized onto a redox-active clay [a composite of $Zn_2Cr(OH)_6$ with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)]. In pristine samples, the laccase continually oxidizes the ABTS as a substrate, but, on contact with respiratory inhibitors, including azides, fluoride, or cyanide, the native laccase activity decreases, thereby indicating the presence of one or more of these toxins.[54]

2.3. Enzymatic fuel cells

The laccase-catalyzed four-electron reduction of oxygen is readily applicable to the cathode reaction in biological fuel cells.[55] In fuel-cell design, the laccase must accept electrons from the cathode in some manner and a number of alternative approaches to facilitate this redox process have been reported.[14,56–58] Ideally, direct electron transfer (DET) between the electrode and the enzyme would occur because it would eliminate the need for redox mediators and their associated overpotential losses in the half-cell reactions. However, for DET to occur, the enzyme must be immobilized onto the electrode with its active site within electron-tunneling distance and, ideally, organized in a close-packed monolayer. Enzymes on top of the initial monolayer would impede the diffusion of the reactants (including oxygen) to the active enzymes that are electronically connected to the electrode. A connection between the enzyme and the electrode can be achieved by combining various methods, including: 1) Physical adsorption, 2) electrostatic interactions, and 3) covalent bonding. For example, a stable laccase monolayer can be immobilized onto a carbon electrode and then fixed through glutaraldehyde cross-linking.[59] The bioelectrocatalytic activity of the immobilized laccase and DET were confirmed by the effective reduction of dioxygen at a redox potential that was equivalent to that of the laccase T1 copper center. In contrast, the bacterially expressed small laccase (SLAC) of *Streptomyces coelicolor* demonstrated optimal mediated-electron-transfer kinetics if the anionic enzyme molecule was incubated so as to form an electrostatic adduct with the cationic mediator.[59]

Covalent bonding positions the enzyme directly on the electrode surface by using chemical techniques to link specific amino-acid residues of the enzyme onto the electrode.[60] Such immobilization methods have been successfully employed for laccases by using thiol-rich compounds, such as α-lipoic acid and 4-aminothiophenol, to activate gold surfaces.[60,61] In the former case, the enzyme was chemically tethered onto a nanoporous gold electrode by modifying the gold surface with Au–S bonds. The activated particles were placed into contact with laccases and the thiol-esters interacted with the surface amines of the protein. Following esterification, *N*-hydroxysuccinimide groups were replaced by an amino group on the surface of the protein. This replacement resulted in an immobilized laccase that was chemically bonded to the gold surface and in close-enough proximity to allow for electron tunneling. Interestingly, this covalent-coupling method may have been further enhanced by the specific interactions between laccase and nanoporous gold. Laccase of *T. versicolor* contains eight lysine residues, four of which are on (or near) the surface and can conceivably supply a bridge to the nanoporous gold through the N terminus of the protein side-chain. Michaelis–Menten-type kinetic analysis by Qiu et al.[60] revealed that specific sizes of the nanoporous gold particles (μm size) improved

the catalytic efficiency of laccase, possibly by positioning the active site in a manner that enhanced mass transfer.

Laccases can use a variety of aminophenols as substrates and this binding affinity can be exploited by using substrates (and substrate mimics) to anchor proteins onto electrode surfaces through selective covalent binding. For example, laccase from *T. hirsuta* was covalently linked onto graphite by modifying the surface with 2-aminophenol; current densities of up to 0.5 mA cm^{-2} were obtained in the absence of redox mediators and the enzyme activity was was insensitive to chloride inhibition.[62] Similarly, Pang et al. reported a method to functionalize CNTs with 1-aminopyrene, which helped the dispersivity of the CNTs and also provided the stable immobilization of laccase, as evidenced by its bioelectrocatalytic activity for oxygen reduction.[58] The aromatic moiety of the aminophenyl group interacts with CNTs through π–π stacking and the amino group can be linked to the protein by using glutaraldehyde-binding chemistry. However, the resulting current density was relatively low (about 20 μA cm^{-2} at pH 7.0) compared to values that were achieved by using alternative techniques.

The substrate specificity of laccase also extends to polycyclic aromatic hydrocarbons. Blanford et al. reported an efficient method for enzyme immobilization through affinity interactions with anthracene on modified pyrolitic graphite with the aim of specifically directing the enzyme to bind in the hydrophobic substrate-binding pocket (the T1 copper site).[63] By using this approach, the electrocatalytic activities of two laccases from *T. versicolor* and *Pycnoporus cinnabarinus* were demonstrated with current densities approaching 0.5 mA cm^{-2} and long-term stabilities of up to 8 weeks.[63] From crystallographic studies of various laccases, such as *T. versicolor* laccase, it is known that the T1 site is surrounded by a negatively charged pocket, owing to carboxylate residues, which are deprotonated at pH values above 5.[64] Therefore, electrostatic interactions influence the orientation of the immobilized laccases and fortuitously position the protein such that the T1 site faces the electrode.

3. Heterogenization of laccases

There are a wide variety of techniques that are available for the immobilization of proteins and many of these show good catalytic activity for the stabilization of laccases. Fernández-Fernández et al. reported an excellent summary of the various methods for the immobilization of laccases and the reader is referred to that review article for further details.[65] The strategies discussed herein are restricted to applications of heterogenized laccases in which electrochemical connectivity is critical, and to applications that demonstrate how immobilization chemistry can significantly affect bioelectrocatalytic activity.

For example, Tortolini et al. derivatized multi-walled CNTs (MWCNT) to provide three different surface functionalities for laccase immobilization: 1) Physico-chemical entrapment inside a polyazetidine pre-polymer (PAP), 2) electrostatic attractions (within Nafion), and 3) covalent linkage by using *N*-hydroxysuccinimide and carbodiimide chemistry. Next, they tested the differences in laccase activity on each material. Laccase that was adsorbed onto PAP-modified MWCNTs exhibited optimal catalytic activity, which was attributed to preferential immobilization of the active site with respect to substrate accessibility. In addition, the PAP-modified MWCNTs demonstrated higher sensitivity of detection for two model substrates: ABTS and catechol.[66] Calculation of the diffusion coefficients across the respective coating materials indicated that the observed difference in sensitivity was attributed to increased mediator permeability in the presence of negatively charged groups on the support matrix. This result demonstrates how the choice of immobilization chemistry requires some consideration of the final application and operating conditions. For example, improvement in catalytic properties upon physical adsorption may be related to specific enzyme and support interactions, but this, in turn, is dictated by the use of supports that are compatible with the desired application. Therefore, the immobilization strategies summarized herein are intended to demonstrate the "toolbox" of available techniques, depending upon individual design considerations, rather than a single method that will work in all cases.

3.1. Adsorption

The adsorption of proteins involves the physical association of an enzyme onto a support through electrostatic, hydrophobic, or hydrophilic interactions; these interactions do not require complex surface-derivatization procedures and, therefore, provide one of the simplest methods for immobilization. This fact is evidenced by the large number of biosensors that have been fabricated by the physical adsorption of enzymes onto various supports (Table 1). Moreover, this technique has the advantage of reversibility, thus allowing the reuse of the electrode after the enzyme has been inactivated.[67,68] The reversibility of laccase adsorption onto different supports was demonstrated by Durmaz et al. with differently functionalized polydivinylbenzene microspheres. In that work, the authors used zeta potentials as an indicator of the surface charge on the support particles. In comparing the advantages of adsorbing the laccase from *Agaricus bisporus* onto purely hydrophobic, hydrophilic, or mixed surfaces, they demonstrated reversible adsorption onto—and desorption from—functionalized surfaces.[69] Optimal protein adsorption was observed on poly(methyl-methacrylate)-functionalized microspheres, which allowed for mixed hydrophobic and hydrophilic interactions that stabilized laccase in an active conformation.

In some cases, adsorption confers additional stability against denaturation and provide a protected microenvironment that leads to enhanced catalytic properties of the enzyme.[70] For example, Labus et al. demonstrated the reuse of adsorbed *Cerrena unicolor* laccase (10 times) on polyamide membranes with no loss of activity. Interestingly, the covalent attachment of the enzyme through amine, hydroxy, or carboxy groups did not provide any significant improvement in the activity or stability of the enzyme over immobilization through physical adsorption.[71]

The leakage (disassociation) of enzyme molecules is the primary disadvantage of immobilization by adsorption and is the

most common argument for choosing covalent immobilization over adsorption, regardless of the typical decrease in activity that accompanies covalent interactions.[72] If adsorption occurs through electrostatic interactions, immobilization efficiency can be controlled by optimizing the ionic strength and pH value of the enzymatic environment to prevent enzymatic desorption.[73,74] However, multiple interactions govern physical adsorption and these are less predictable if the reaction conditions change.[73]

Portaccio et al. compared physical adsorption to covalent immobilization for laccase from *T. versicolor*. Covalent binding was achieved by functionalizing the surface, either by applying a specific potential or by treating with nitric acid to create carboxylate functional groups on the electrode surface.[75] The sensitivity of the physically adsorbed laccase was poor (10 to 50-fold lower) compared to electrodes that were prepared by covalent binding and their long-term stability was lower compared to non-encapsulated enzymes, as a result of desorption of the protein over time.

3.2. Stabilization through encapsulation

Encapsulation typically protects an enzyme from the surrounding operating environment, but mass transfer of the substrate can be limited compared to non-encapsulated enzymes. Microencapsulation overcomes some of these limitations by confining the biocatalyst within the core of micron-sized particles that are made from semi-permeable materials, such as polymers (e.g., polyethyleneimine) or inorganic materials (e.g., SiO_2). In particular, encapsulation inside silica sol–gels has been found to be effective for laccase stabilization and, despite the loss of unit activity, changes in catalytic activity have been observed. For example, a change in the optimum pH value for the oxidation of 2,6-dimethoxyphenol by laccase from *Trametes* sp. was observed following immobilization by encapsulation; this change was attributed to changes in proton partitioning at the active site, owing to the charge on the encapsulation matrix.[76] Similarly, laccase from *Cerrena unicolor* was immobilized by sol–gel fabrication with graphite particles; redox-mediator ABTS was co-immobilized inside the silica to enhance the electrocatalysis.[77]

Layer-by-layer (LbL) is a technique in which encapsulation is used to create functional thin films on electrode materials, such as gold. Scodeller et al. encapsulated laccase from *T. trogii* by using a LbL method to coat gold electrodes. The LbL films were fabricated by the sequential immersion of a functionalized gold electrode into laccase and a polyelectrolyte mediator (osmium complex of poly(allylamine)).[57] However, in this case, the catalytic activity of the resulting laccase was limited and hydrogen peroxide was observed as a byproduct, owing to incomplete oxygen reduction.[57] Similarly, *T. trogii* laccase was deposited along with mercaptopropanesulfonate by sequential immobilization onto a gold electrode and, again, the electrode was coated with an osmium complex that was derivatized with polyallylamine.[78] An increase in the catalytic current was observed on increasing the number of layers of redox-mediator polyelectrolyte and enzyme that were deposited onto the electrode, until the mass transfer became limited. The resulting electrode also exhibited increased stability toward MeOH and chloride. Gallaway and Barton (2008) used a similar approach with a series of osmium-based redox polymers and reported catalytic current densities of up to 2.5 $mA\,cm^{-2}$ for immobilized *T. versicolor* laccase.[79]

A potential limitation of encapsulation as a heterogenization strategy is the physical separation of enzyme–mediator or enzyme–electrode interactions, which can prevent the effective shuttling of electrons between the enzyme and the substrate. As a result, whereas enzyme loading is typically high with entrapment strategies, activity can be significantly reduced. In general, a monolayer of enzyme molecules that covers the electrode is more beneficial to electron transfer than embedding the bulk protein inside a thick layer of insoluble material, which can limit electron transfer and substrate accessibility. For example, the encapsulation of laccase from *Coriolopsis gallica* inside a carbon/Nafion composite ink resulted in an enzyme loading of $>1000\ \mu g\,cm^{-2}$, but the catalytic activity for ABTS oxidation was almost 40-times slower than for a covalently attached protein with a comparatively low enzyme loading (about 2 $\mu g\,cm^{-2}$).[80]

The electropolymerization of pyrrole is another commonly used method for immobilizing redox enzymes. In one example, laccase that was entrapped inside a polypyrrole matrix on an electrode surface effected the catalytic reduction of oxygen in the presence of ABTS.[81] Although the enzyme loading was high in the pyrrole matrix, laccases that were covalent grafted onto an aminopolypyrrole film through glutaraldehyde cross-linking provided a twofold increase in enzyme activity compared to encapsulation (20–40 $mU\,cm^{-2}$ and 70–90 $mU\,cm^{-2}$, respectively).[81]

3.3. Covalent attachment

A plethora of covalent-attachment methods have been successfully used to integrate laccases into electrodes and transducer interfaces for various devices. Such methods usually require several steps for the functionalization of the electrode surface, but they usually yield stable preparations that perform efficiently over extended periods of operation. The possibility of enzyme-supported covalent interactions is dictated by the availability of amine, hydroxy, sulfhydryl, and carboxylate groups on the surface of the enzyme. The amino-acid side-chains are coupled to interfaces that have been functionalized by using complementary chemistry to tether the biocatalyst, without necessarily changing the physical characteristics of the material.

For example, glassy carbon electrodes can be modified with phenyl–NH_2 moieties by electrochemical reduction and the modified electrodes can be used to stably heterogenize CotA (a multicopper oxidase) from *Bacillus subtilis* as an oxygen-reduction catalyst. CotA was immobilized by cross-linking a peripheral amine group on the protein with the phenyl–NH_2 moieties on the glassy carbon surface.[12] CotA is a thermophilic protein with an optimal temperature in the range 45–50 °C and an operating range of up to 75 °C; this result significantly

extends the application of this enzyme to operating conditions that most laccases will not tolerate. Interestingly, oxygen reduction was observed to increase almost twofold with temperature, as the enzyme approached its optimal activity range.

Similarly, Rahman et al., immobilized laccase by forming amide bonds with terminal carboxylic-acid groups on gold-nanoparticle-encapsulated dendrimers.[82] The electrical properties of the sensor surface were enhanced by confining the gold nanoparticles to the interior of the dendrimer and, thus, controlling the size of the formed nanoparticles. This approach showed improved catalytic stability compared to colloidal gold nanoparticles and enhanced DET with immobilized *Rhus vernicifera* laccase. The resulting biosensor was used for the detection of catechin in green tea.

Methods to covalently functionalize an electrode surface primarily depend on the nature of the electrode material. For example, glassy carbon can be carboxylated by the electrochemical oxidation of 1,5-pentanediol on the electrode surface or by the direct electrochemical oxidation of the glassy carbon material. Then, the carboxy groups can be modified by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) chemistry to covalently bond to amino-acid side-groups on the laccase. Interestingly, the direct electrochemical method provides higher coverage of surface carboxylate groups, but the response time of the resulting laccase-bound biosensor is much faster following the electrochemical modification of 1,5-pentanediol.[83] Similarly, electrochemical oxidation was used to introduce hydroxy groups onto platinum, which allowed the enzyme and the electrode to be coupled by using cyanuric chloride chemistry.[83] Surfaces can also be activated by using silanization treatments that allow for glutaraldehyde and laccase cross-linking.[86] Typically, covalent immobilization stabilizes the enzyme but also enhances the feasibility of certain applications, such as flow-through process streams. For example, laccase from *Rigidoporus lignosus* was covalently immobilized by using carbodiimide chemistry onto a self-assembled monolayer (SAM) of 3-mercaptopropionic acid that was deposited on a gold surface. The biosensor was stable under flow conditions and was used in the detection of phenols in olive mill waste streams.[84,85]

3.3.1. Cross-linked enzyme aggregates

Cross-linked enzyme aggregates (CLEAs) represent an alternative to conventional immobilization on solid supports. CLEAs depend on cross-linking of the enzyme itself, but the approach is rather fastidious; optimization of procedures is required in each case, even for the same family of enzymes that are obtained from different sources. One example of a laccase CLEA involved *Coriolopsis polyzona* laccase that was cross-linked by using glutaraldehyde as the aggregating agent.[87] Similarly, Matijosyte et al. studied and optimized the preparation of laccase from *T. versicolor*, *T. villosa*, and *A. bisporus*. In each case, the cross-linked aggregates were stable and reusable and had the additional advantage (inherent to CLEAs) of high volumetric activities.[88] In that case, the activity of phenol oxidase was retained, but the electrocatalytic activity of such composites has not been reported for laccases. It would be particularly challenging for developers to use CLEAs as electrocatalysts in DET, but such processes may be amenable to mediated transport regimes.

3.4. Molecular tethering

The research developments into—and increased availability of—CNTs in recent years has matched parallel attempts to integrate conductive CNT architectures into general electrochemical systems and, specifically, into biosensors.[89] However, productively integrating proteins and CNTs is not trivial and relies on the selective orientation of the proteins on the surface of the CNTs, without decreasing the electronic conductivity of the bulk matrix. By using specific binding chemistry, preferential binding can shorten the distance between the protein and the CNT surface and, thus, optimize the electronic connectivity. CNTs can be functionalized by using a variety of techniques to impart amine, carboxylic acid, or hydroxy groups onto the CNT surface, all of which provide specific moieties for the immobilization of proteins. By varying the type of functionalization chemistry that is used, proteins can be immobilized at specific or strategic tethering points (Figure 5). In the simplest case, laccases may be immobilized onto the CNT surface by the physical adsorption of the protein onto the hydrophobic CNT surface, largely through van der Waals forces; however, these weak noncovalent interactions are typically labile. The most common method for covalent heterogenization uses chemical oxidation of the CNTs to yield carboxylic-acid functional groups that can react with amine groups on the protein surface to form covalent amide bonds. However, the oxidation of CNTs creates defects in the surface, which lower the conductivity of the material. Moreover, a short covalent bond can force steric constraints on the protein structure that decrease catalytic activity.

Laccase can be immobilized on—and electronically linked to—CNTs through simple physical adsorption, but its heterogenization must be significantly improved for practical use. A better method for enabling connectivity would activate the surface for protein interactions but not change the conductivity. One such approach uses 1-pyrenebutanoic acid succinimidyl ester (PBSE), a bifunctional cross-linker, in which the pyrene moiety interacts with the CNTs through strong π–π stacking interactions and the succinimidyl ester group is available to link with the protein through covalent amide bonds. Directed orientation is provided by accessible primary and secondary amines, which are exposed on the protein surface. This process results in the heterogenization of laccase and the CNTs and provides stable electrocatalytic activity with cathodic current densities that are applicable to the development of enzymatic fuel-cell cathodes.[90] This approach is also amenable to the treatment, activation, and functionalization of CNT paper, known as "buckypaper". The material profile of buckypaper facilitates the scaling of the process and its integration into electrochemical applications.[90]

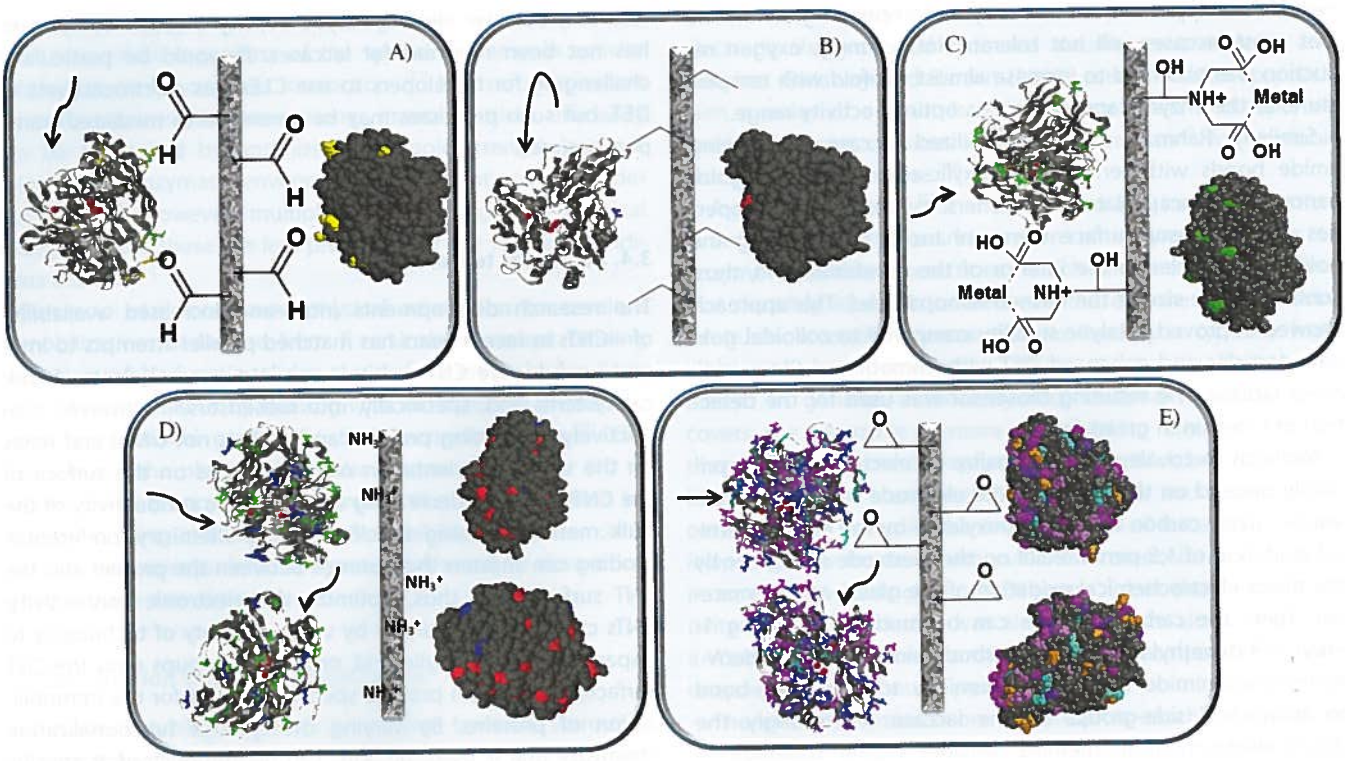

Figure 5. Left: Ribbon view, which shows the position of the T1 and T2/T3 copper centers (red spheres) in relation to the electrode surface. Access to the T1 binding pocket is marked by an arrow. Right: Solid-fill view, which shows the positions of the amino-acid side-chains that react with specific supports: Glyoxyl-functionalized support (A), glutaraldehyde-functionalized support (B), metal-chelate support (C), amino-activated support (D), epoxy-activated support (E). Key: Asp (red), Glu (blue), Ser (purple), Asn (violet), Thr (magenta), Gln (blue), Arg (cyan), His (green), Lys (yellow). The amino terminal in Image B is shown on the left side in blue and on the right side in red.

Chitosan matrices have also been used as another architecture in which laccase and CNT can be combined without the need for complex functionalization chemistry.[67] Laccase from *T. versicolor* that was immobilized onto glutaraldehyde-functionalized chitosan, with ABTS as a redox mediator, exhibited strong electrocatalytic activity that was attributed to the electrochemically active ABTS in the composite film. The current density for laccase electrocatalysis increased significantly on including MWCNTs in the chitosan/ABTS composite.[91] The resulting material showed strong oxygen-reduction activity in the presence of ABTS and was coupled with a glucose bioanode to afford an enzymatic biological fuel cell.[91] The same heterogenization method also provided a stable laccase-based catalyst for the detection of catechol; the detection threshold for catechol was 20 nM and 85% of the initial activity was sustained for over 1 month.[67]

4. Orientation matters

A recent trend in protein-immobilization research is the design of rational approaches for controlling the specific orientation of enzymes with respect to the interface between the materials on fixing biomolecules onto a selected surface.[92,93] This strategy aims to create uniformly oriented proteins that are immobilized so as to optimize their catalytic efficiency (through better substrate accessibility)[94] and to enhance their stability (by fixing the protein structure in a particular position).[93] With redox enzymes, such as laccases, the aim is more specifically to increase the efficiency of DET to an electrode surface.[95] Oriented immobilization may be achieved by introducing reactive groups or tethers onto the protein surface that interact with compatible functionalized surfaces (Figure 5). Alternatively, careful control of the immobilization conditions can exploit the inherent physicochemical properties of the enzyme.[93–96]

Several reports have concluded that the orientation of laccase affects its electrocatalysis and electrode performance;[97] however, to date, there have only been a few reports of the effective attachment of laccases at a particular position or in a particular orientation. In one example, Pita et al. developed a strategy that covalently immobilized *T. hirsuta* laccase onto gold electrodes.[95] After optimizing the immobilization to enhance DET and the reduction of the T1 copper site, they measured current densities of 40 $\mu A\,cm^{-2}$ for the electrocatalytic reduction of O_2. In another report, tethering the laccase onto a gold surface diminished the reduction of O_2, thus demonstrating that heterogenization is not always preferential. Szamocki et al. immobilized *T. trogii* laccase onto gold surfaces by modifying the gold surface with dithiobis(*N*-succinimidyl propionate); the thiol groups bound to the gold surface and, then, the protein was bound through reactions with the amine groups on the protein surface. Subsequent analysis of the immobilized laccase films by using cyclic voltammetry showed poor oxygen reduction in the absence of redox mediators. Instead, it appeared that the link between the protein and the

electrode was such that electron transfer was directed towards the T2 copper site, thereby bypassing the T1 site and eliminating the electrocatalytic reduction of oxygen.[78] This result illustrates how the positioning and orientation of an enzyme may influence its electrocatalysis, in particular for DET.

The distance between the catalyst and the electrode may be controlled by using a linker structure, which could be used to influence the electron transfer. Laccase from *Cerrena unicolor* was immobilized onto a gold electrode by covalent attachment with various alkanethiol SAMs. Changing the length of the SAM spacer molecules from mercaptoundecanoic acid to mercaptopropionic acid significantly increased the catalytic activity of the electrode towards oxygen reduction, thus suggesting that electron transfer between the enzyme and the electrode was enhanced with shorter spacer molecules.[98] In further studies, a metal ligand and a modified protein were used to functionalize a gold electrode. In that case, thiolated nitrilotriacetic acid (NTA) was used to activate the gold surface, the thiol group again bound to the gold surface, thereby forming a SAM, and the NTA chelated to the nickel center, thus providing a ligand for proteins that had been modified with hexa-histidine linkers (his-tag). The activity of a his-tag-modified laccase on a gold surface was examined to reveal any specific protein binding with the metal ligand and it was found to result in the formation of a protein monolayer on the electrode surface and the retention of catalytic activity.[99] The retained activity shows promise as an approach for electrode assembly, but the ability to influence protein orientation by using his-tag linkages is limited because only the N or C terminus may be modified; the other domains of the mature protein would still be the primary determinant of the overall interaction with the electrode surface.

A detailed understanding of the enzyme structure would undoubtedly help to develop effective strategies for linking enzymes onto surfaces. For example, laccase from *Coriolopsis gallica* has seven reactive surface lysine groups that may link onto an activated graphite surface by using carbodiimide chemistry. None of these reactive lysine groups are close to the substrate-binding and associated T1 copper sites. Accordingly, direct binding will presumably not orient the enzyme to facilitate DET. Martinez-Ortiz et al. modified a graphite surface with a substrate mimic for laccase, 4-(2-aminoethyl)benzoic acid hydrochloride, with the intent of biasing the orientation of the electrode surface toward the enzyme's substrate-binding site to improve DET. The resulting laccase-immobilized electrode was used in a semi-enzymatic fuel cell (with zinc as a co-catalyst). Calculations indicated that the optimized orientation provided increased volumetric catalytic activity, that about 26% of the laccase molecules were preferentially oriented towards the T1 active site, and that this ordered binding produced a cathode that showed 37% higher power density and 43% higher current density than the "randomly" bound laccase cathode.[80]

5. Entrapment as a pre- and post-immobilization strategy

The benefits of diverse pre- and post-immobilization treatments upon the activity and stability of biosensing enzymes have long been known.[100,101] These treatments can include chemical modification or the "caging" of immobilized enzymes within polymers. In general, these stabilization strategies prolong the half-life of the enzymes, prevent enzyme loss, and/or avoid decreased activity owing to constraints on the enzyme molecules within the support. These advantages usually compensate for the additional steps that are required for their fabrication. One approach, which was demonstrated in 2012 by Tortolini et al., embedded *T. versicolor* laccase inside a polyazetidine pre-polymer and then deposited the composite mixture onto MWCNTs, thereby forming a catalytic conductive architecture. Compared to covalent and electrostatic immobilization on the same MCWNTs, the physical-chemical-deposition approach better preserved the activity of the enzyme during immobilization and had a higher sensitivity towards diffusible electron donors, including ABTS, catechol, dopamine, and caffeic acid.[66] Similarly, *T. versicolor* laccase can be microencapsulated in polyethyleneimine (PEI) prior to coating onto a paper support; although initial reports demonstrated lower laccase activity after PEI immobilization, owing to negative conformational changes,[102] optimized coating conditions showed that microencapsulation had a stabilizing effect compared to the non-encapsulated enzyme.[101]

6. Outlook

The field of enzyme immobilization, though maturing after years of investigation, still offers no universal procedure for every enzyme system and the application of laccases in bioelectrocatalysis is certainly no exception. However, over the last five years, a number of methods have been reported that have started to establish a toolbox for the functionalization of materials. These advances provide high volumetric activity, good stability, and bioelectrocatalytic activity that support laccase-catalyzed electrochemistry. We anticipate that subsequent contributions to the field will lead to various technological applications, such as in fuel cells and sensors, but also in the development of molecular-screening tools for biomarkers in diseases, including Down syndrome and Parkinson's disease.[40,103] Primary challenges involve the establishment of efficient electronic connections between the enzyme and the electrode, along with stabilizing and sustaining of catalytic activity to facilitate the application of the concepts. After building an understanding of catalytic complexity and the laccase structure, it is clear that immobilization strategies that orient the protein structure may heavily influence their use in biocatalytic applications, particularly with respect to electrocatalysis.

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